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OPTIMIZATION STUDIES FOR CLOZAPINE BIOTRANSFORMATION TO ITS ACTIVE METABOLITE - NORCLOZAPINE USING FUNGI.

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ABSTRACT

Clozapine is an antipsychotic drug which is used for treatment of schizophrenia. It is metabolised by CYP1A2 enzyme to its active metabolite norclozapine in human beings. The biotransformation of clozapine was performed by eight strains of fungi using fermentation technique and HPLC analysis. Among which a filamentous fungus *Cunninghamella elegans* has shown ability to biotransform clozapine to its active metabolite, norclozapine which was identified by HPLC, Mass spectroscopy and ^{1}HNMR studies. Then the influence of different parameters like change in media components such as carbon and nitrogen sources used in media, incubation period and agitation speeds on metabolite production was investigated and also their respective cell dry weights were determined during optimization studies. Among different carbon and nitrogen sources used, maximum metabolite was formed when dextrose and yeast extract were used as carbon and nitrogen sources respectively. The influence of different concentrations of dextrose and yeast on the metabolite production was also investigated. Maximum biotransformation was observed at 2% dextrose, 0.001% yeast extract, 48th hour of incubation period and at 120 rpm. Based upon the results, it can be concluded that there is an influence of media components and their concentration, incubation period and agitation speeds on extent of biotransformation of clozapine to its active metabolitenorclozapine by fungus. Optimized conditions were determined in present study for maximum production of norclozapine.

KEYWORDS: Clozapine, fungal biotransformation, active metabolite, norclozapine, *Cunninghamella elegans*.

INTRODUCTION: Drug biotransformation is defined as the transformation of drugs from one chemical form to other form which occurs mainly by specialised enzymatic systems. Biotransformation involves conversion of lipophilic compounds [drugs] into highly polar derivatives which can be easily excreted out of the body. Hence Drug metabolism can also be termed as a detoxification mechanism.[1,2] Drug metabolism and toxicity studies of metabolites plays a vital role in drug development. Identification of the metabolites from clinical samples and animal sources is hindered by minimum quantity of material obtained.[3] Microbial biotransformation involves the chemical transformation of substrates to desired products by using microorganisms [bacteria and fungus].[4,5] Recently Microbial biotransformation is majorly used for the creation of novel and useful metabolites of almost all classes of drugs which is a substitute for chemical synthesis to prepare pharmacologically active compounds.[6] Among different microorganisms the fungi belonging to *Cunninghamella* species, possess cytochrome P-450 monooxygenase systems which is similar to mammals. Hence microbial biotransformation studies have been proposed as a complementary *in vitro* model for mammalian drug metabolism.[3] One of the advantages of microbial models is the production of milligram quantity of metabolites by preparative-scale fermentation for complete structural elucidation and for further pharmacological and toxicological studies with minimum cost.[7-9] Clozapine is an atypical or new generation antipsychotic drug.It is mainly used in individuals with schizophrenia who are either resistant or intolerant to other antipsychotic drugs[10].It is metabolised by CYP1A2 enzyme to its active metabolite norclozapine in human beings.[11] Thus the research work was carried out to screen different microorganisms for metabolism of clozapine to its active metabolite norclozapine as synthesis of metabolites by other methods exposes to number of obstacles. An effect of different conditions on the metabolite production was performed using different carbon and nitrogen sources at different concentrations, incubation period and agitation speeds.

MATERIALS AND METHODS

Microorganisms

Aspergillus flavus [MTCC 1783], *Aspergillus ochraceus* [NCIM 1140], *Aspergillus terreus* [NCIM 657], *Cunninghamella blakesleeana* [MTCC 3729], *Cunninghamella echinulata* [NCIM 691], *Cunninghamella elegans* [NCIM 689], *Gliocladiumroseum* [NCIM 1064] *Rhizopusstolonifer*[NCIM 880] were procured from National Chemical Laboratory [NCL] Pune, India and microbial type culture collection [MTCC] Chandigarh, India.

Chemicals

Clozapine was obtained as gift sample frommylan laboratories, Hyderbad, India. All the solvents used for analysis were HPLC grade. The remaining chemicals and culture media components were purchased from Qualigens & S.D. fine chemicals, Mumbai, India.

Fermentation technique

The fermentation technique was carried out in 250ml Erlenmeyer flask containing 50ml of broth media containing Potato chips [20gm/100ml, steamed for 30 min], dextrose 2gm, yeast extract10mg, distilled water upto 100ml [final pH 5.6]. Study consisted of one drug control which has drug and incubated without organism, culture control consisted of broth medium inoculated with a loopful of respective fungi. Sample flask consisted of both drug and culture. Two controls and sample flasks were incubated on orbital shaker under identical conditions to obtain the prominent growth of microorganisms for biotransformation study.

Extraction procedure

The incubated flasks were taken out from shaker incubator and heated on water bath at 50°C for 30 min for inactivation of grown microbes. Then, these were transferred into centrifuge tubes and centrifuged at 3000 rpm for 10 min [R8C: Remi instruments, Mumbai, India]. The supernatant obtained was collected in separate boiling tubes. Drug and its metabolitewere extracted from the supernatant by usingethylacetate.[12] Then organic layer was collected and air dried. The dried extract was reconstituted with mobile phase for HPLC analysis.

Optimization studies

Optimization studies were conducted to find the most suitable conditions to be maintained during fermentation for obtaining maximium metabolite by fungal biotransformation. These studies were performed using the fermentation procedure explained as above and by changing the incubation conditions like composition of media, incubation period and agitation speeds as shown in Table 1. All the samples collected during optimization studies are also extracted and analysed by HPLC similarily. The percentage metabolite formed was calculated from the peak area of metabolite obtained during HPLC analysis.

ANALYTICAL METHODS

High pressure liquid chromatography

Clozapine and its metabolite in the extracted samples during screening and optimization studies [as shown in Table 1] were estimated by High Performance Liquid Chromatography

[HPLC] method. The HPLC system [Waters, USA] consisted of Waters 515 solvent delivery module and Waters 2489 UV-visible spectrophotometric detector. The mobile phase comprised of Sodium Sodium acetate buffer [pH – 5] [50:40:10v/v] with a flow rate of 1ml/min. The column used was C-18 [stainless steel column of 25 cm length and 4.6 mm internal diameter packed with porous silica spheres of 5 µ diameter, 100 Å pore diameter – II 5C-18 rs – 100a, 5 µm, 4.6 x 250 mm]. The wavelength was set as 250nm and sensitivity at 0.001 a.u.f.s.[13] The percentage of metabolite was calculated based on the area of peak obtained in HPLC analysis and percentage of metabolite formed under different optimized conditions was calculated and compared.

Determination of biomass production

The biomass production at different conditions was determined as it was correlated with the quantity of metabolite produced under same conditions by the following procedure. An empty eppendroff tube is weighed [w_1]. Fixed amount of filtered suspension cell material is added into the eppendroff tube. The eppendroff tube [cell material] is weighed again[w_2]. The eppendroff tube with the cell material is placed into an oven at 60 °C until consecutive readings are obtained. The eppendroff tube [including dried cell material] is weighed again [w_3]. cell dry weight was calculated by using below formula.[14]

Cell dry weight = $[w_3-w_1] / [w_2-w_1]$.

RESULTS

The HPLC analysis of clozapine and its metabolite in different culture extracts at different conditions was conducted. The peak at retention time of 2.2min represented solvent peak, peak at 4.2min represented culture content and peak at retention time of 8min represented clozapine and interestingly the sample of *Cunninghamella elegans* has shown an extra metabolite peak at 3.5 min compared to its controls as shown in Figure.1. It is indicated that *Cunninghamella elegans* has metabolised the drug. The structure of metabolite was analysed and confirmed as norclozapine by Mass spectroscopy and ^{1}HNMR techniques. Then the extent of this metabolite formation by *Cunninghamella elegans* at different optimization conditions was determined as shown in Table 1.

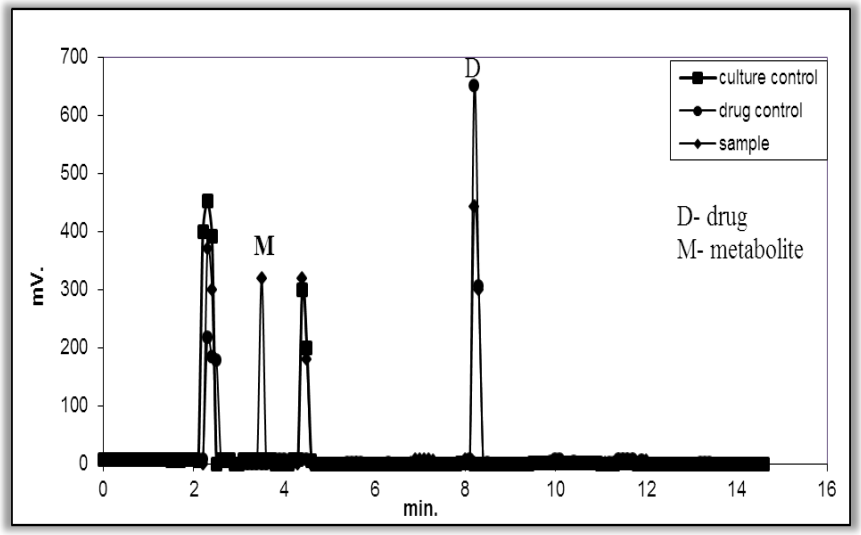

Figure1. HPLC chromatogram of clozapine from culture extracts of *Cunninghamella elegans*

Table 1: List of optimization parameters studied and their results.

Optimization parameters	% Metabolite	Cell dry weight [mg/50ml]
Carbon sources		
Dextrose	**73***	**23.68***
Ribose	64	20.60
Starch	42	13.49
Nitrogen sources		
Ammonium acetate	68	22.01
Ammonium chloride	65	21.04
Calcium nitrate	61	19.65
Barium nitrate	54	16.98
Urea	57	18.47
Yeast extract	**73***	**23.80***
% of dextrose		
1	69	22.25
2	**73***	**23.66***
3	70	22.68
4	68	21.82
5	60	19.41
6	56	17.99
% of yeast extract		
0.005	63	20.36
0.01	**73***	**23.67***
0.02	57	18.47
Incubation periods[hrs]		
6	0	0
12	3	1.6
24	24	7.5

48	**73***	**23.01***
60	61	19.65
Agitation speeds[rpm]		
60	35	11.12
80	42	13.49
100	60	19.41
120	**73***	**23.12***
160	48	15.39

"*" – Maximum % of metabolite and cell dry weight formed.

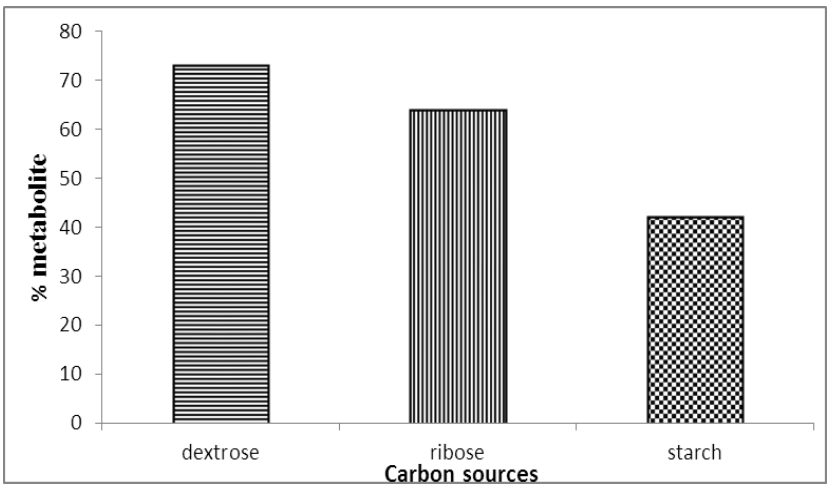

Figure 2: Graphical representation of influence of different carbon sources on metabolite production.

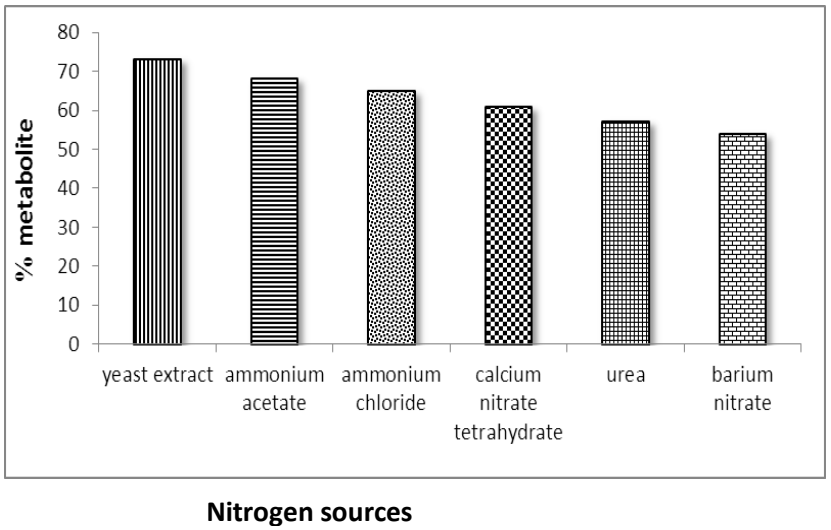

Figure 3: Graphical representation of influence of different nitrogen sources on metabolite production

Dextrose and yeast extract were selected as the best carbon and nitrogen sourcesbased on percentage metabolite formed in the above results. Then the effect of their concentration on metabolite production so as to optimize the conditions that favours the maximum growth of

Cunninghamella elegans collect the norclozapine[metabolite]in large amounts was studied and results are shown in Table 2 and in Figure 2, Figure 3.

Table 2: Effect of concentration of carbon and nitrogen sources on formation of metabolite.

Concentration of carbon and nitrogen sources	% metabolite	Cell dry weight [DCW]mg/50ml
% of dextrose		
1	69	22.25
2	**73***	**23.66***
3	70	22.49
4	68	22.02
5	60	19.41
6	56	17.99
% of yeast extract		
0.005	63	20.36
0.01	**73***	**23.67***
0.02	57	18.47

"*" – Maximum % of metabolite and cell dry weight formed.

DISCUSSION

The production of metabolites of drugs by microbial biotransformation process may vary with different environmental conditions and media composition.[15,16] It was also reported that production of the metabolites by microorganisms was influenced by the media composition and different concentrations of carbon and nitrogen sources used.[17,18]

The effect of different carbon and nitrogen sources on the metabolite production and its cell dry weight were determined as the media components influence the physical and chemical integrity of the cells of microorganisms.[14] The percentage yield of metabolites can be increased by optimizing the physical and chemical factors which effect the growth of microorganisms.[19-28]

The influence of carbon and nitrogen sources and change in concentration of these sources on metabolite formation and cell dry weight indicated the existence of relationship between percentage of metabolite production and growth of organism, *Cunninghamella elegans*. Maximum production of metabolite i.e. 73% and its cell dry weight was 23.67mg/50ml by *Cunninghamella elegans* was found with dextrose compared to other carbon sources used. Similarly among the different nitrogen sources used, yeast extract has shown maximum production of metabolite i.e. 73% and maximum cell dry weight. Dextrose and yeast extract

have shown maximum metabolite production than other carbon sources and nitrogen sources since these are readily absorbable and easily employed by fungi.[14]

Among the selected concentrations of carbon and nitrogen sources, 2% of dextrose and 0.01% of yeast extract shown maximum metabolite production because as the concentrations of carbon and nitrogen sources are increased, the decrease of enzyme production may be due to inhibition at higher concentrations similar to the reports of V. Varalakshmi*et al.*[29]

The microbial biotransformation was carried out for a period of 60 hrs and the samples were withdrawn and analysed at regular intervals. The maximum metabolite production with maximum cell dry weight was found after incubation to 48 hrs. Fermentation above 48hrs showed a decrease in enzyme production, may be due to the inactivation of the enzymes because of the proteolytic activity or the growth of the organism might have reached a lag stage which may no longer balance its steady growth with the available nutrient resources. At higher incubation periods, the decrease in enzyme activity may also be due to depletion of nutrients in broth media and accumulation of end products which are toxic in nature.[29]

In the present study, the maximum metabolite of 73% and cell dry weight of 23.67mg/50ml was found when agitation intensity was kept at 120 rpm. lower metabolite production at higher agitation speeds may be due to breakage of the fungal cell at high speeds and at lower agitation speeds also less amount of metabolite was produced may be due to improper mixing of the medium.[30,31] Different agitation speeds seemed to provide different distribution and transportation of air and nutrients to the cells[32], hence the extent of microbial metabolism of drugs varies based on speed of agitation during fermentation studies.

CONCLUSION

Based on the above discussion, it can be concluded that there is an influence of the media components, incubation periods and agitation speeds on biotransformation of clozapine to its active metabolite by *Cunninghamellaelegans* due to changes in enzyme activity or changes in distribution and transportation of air and nutrients to the cells. From the present results, it was found that the dextrose is the best suitable carbon source compared to other carbon sources at 2% concentration and yeast extract at 0.01% is the best suitable nitrogen source as maximum metabolite i.e. 73% was obtained at 48th hr. of incubation period and 120rpm speed. It can be revealed that *Cunninghamella elegans* can be used as an *in vitro* model for the study of metabolism of Clozapine and also for the production of an active metabolite of clozapine in

maximum yields using the above optimized conditions for further pharmacological and toxicological studies.

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